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February 20, 2013

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001
Phone: (202)564-8940

Subject: Submission regarding the Sensitization Study by Local Lymph Node Assay in the mouse (Draft Report) on the following substances: *fatty amino amide* [redacted], *fatty acid amide* [redacted], *and a fatty acid amine* [redacted] under TSCA Section 8(e)

Dear Sir/Madam:

[redacted] is submitting three draft reports (Report numbers: 41205486, 41205488, and 41205490) of the Sensitization Studies with a fatty amino amide [redacted], a second fatty acid amide [redacted], and a fatty acid amine [redacted] by Local Lymph Node Assay in the mouse pursuant to Section 8(e) Substantial Risk reporting requirements under the Toxic Substance Control Act. The results of these reports indicate that these substances cause skin sensitization. These studies were conducted to estimate the potential of these substances to induce dermal sensitization for SDS and label hazard communication purposes. The SDS for products containing this substance will be updated in accordance with global hazard communication standards.

This submission does contain confidential business information.

Sincerely,

[redacted]

Company Sanitized

harlan®

[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

PROJECT NUMBER: 41205486

AUTHOR: A Sanders

STUDY SPONSOR:

[redacted]

TEST FACILITY:

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases of this study type at least once every three months.

In addition, general facilities are inspected at least once a year and the results are reported to management.

This report has been audited by the Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

§	24 September 2012	Study Plan Compliance Audit
	17 October 2012	Test Item Preparation
	22 October 2012	Test System Preparation
	17 October 2012	Animal Preparation
	18 October 2012	Dosing
	23 October 2012	Assessment of Response
§	24 January 2013	Draft Report Audit
§	Date of QA Signature	Final Report Audit

..... DATE: .....
For the Quality Assurance Unit*

***Authorised QA Signatures:**
Senior Audit Staff: J G Riley BSc (Hons) MRQA, J M Crowther MIScT MRQA, G Wren ONC MRQA, S Bevan BSc (Hons) MRQA, L Blaney MRQA

GLP COMPLIANCE STATEMENT

With the exception noted below the work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

No analysis was carried out to determine the homogeneity, concentration or stability of the test item formulation. The test item was formulated within two hours of being applied to the test system; it is assumed that the formulation was stable for this duration. This exception is considered not to affect the purpose or integrity of the study.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: .....

A Sanders
Study Director

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[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SUMMARY

Introduction. A study was performed to assess the skin sensitisation potential of the test item in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to be compatible with the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 22 July 2010)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

Methods. Following a preliminary screening test in which no clinical signs of toxicity were noted at a concentration of 100%, this concentration was selected as the highest dose investigated in the main test of the Local Lymph Node Assay. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of the undiluted test item or the test item as a solution in butanone at concentrations of 50% or 25% v/v. A further group of five animals was treated with butanone alone. The control group served as a common control with Project numbers 41205488 and 41205490.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
25	7.47	Positive
50	13.16	Positive
100	13.05	Positive

Conclusion. The test item was considered to be a sensitiser under the conditions of the test.

[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION

A study was performed to assess the skin sensitisation potential of the test item in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to be compatible with the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 22 July 2010)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test items that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test item to man.

The study was performed between 01 November 2012 and 05 December 2012.

2. TEST ITEM

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	[redacted]
Description	:	dark orange coloured liquid
Batch number	:	[redacted]
Purity	:	not supplied
Date received	:	18 October 2012
Expiry date	:	18 October 2013
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test item is the responsibility of the Sponsor.

2.2 Preparation of Test Item

For the purpose of the study, the test item was used undiluted and freshly prepared as a solution in butanone. This vehicle was chosen as it produced the most suitable formulation at the required concentration. The concentrations used are given in the procedure section. The vehicle determination record is included as Appendix 3.

The test item was formulated within two hours of being applied to the test system. It is assumed that the formulation was stable for this duration.

No analysis was conducted to determine the homogeneity, concentration or stability of the test item formulation. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

3. METHODS

3.1 Animals and Animal Husbandry

Female CBA/Ca (CBA/CaOlaHsd) strain mice were supplied by Harlan Laboratories UK Ltd., Oxon, UK. On receipt the animals were randomly allocated to cages. The animals were nulliparous and non-pregnant. After an acclimatisation period of at least five days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were in the weight range of 15 to 23 g, and were eight to twelve weeks old.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (2014C Teklad Global Rodent diet supplied by Harlan Laboratories UK Ltd., Oxon, UK) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25°C and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled

by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.2 Procedure

3.2.1 Preliminary Screening Test

Using available information regarding the systemic toxicity/irritancy potential of the test item, a preliminary screening test was performed using one mouse. The mouse was treated by daily application of 25 µl of the undiluted test item to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The mouse was observed twice daily on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Local skin irritation was scored daily according to the scale included as Appendix 4. Any clinical signs of toxicity, if present, were also recorded. The bodyweight was recorded on Day 1 (prior to dosing) and on Day 6.

The thickness of each ear was measured using an Oditest micrometer (Dyer, PA), pre-dose on Day 1, post dose on Day 3 and on Day 6. Any changes in the ear thickness were noted. Mean ear thickness changes were calculated between time periods Days 1 to 3 and Days 1 to 6. A mean ear thickness increase of equal to or greater than 25% was considered to indicate excessive irritation and limited biological relevance to the endpoint of sensitisation.

3.2.2 Main Test

3.2.2.1 Test Item Administration

Groups of five mice were treated with the undiluted test item or the test item at concentrations of 50% or 25% v/v in butanone. The preliminary screening test suggested that the test item would not produce systemic toxicity or excessive local irritation at the highest suitable concentration. The mice were treated by daily application of 25 µl of the appropriate concentration of the test item to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test item formulation was administered using an automatic micropipette and spread over the dorsal surface of the ear using the tip of the pipette.

A further group of five mice received the vehicle alone in the same manner. The control group served as a common control with Project numbers 41205488 and 41205490.

The thickness of each ear of each animal was measured using an Oditest micrometer (Dyer, PA), on Days 1, 3 and 6. Any changes in the ear thickness were noted. Mean ear thickness changes were calculated between Days 1 to 3 and Days 1 to 6. A mean ear thickness increase of equal to or greater than 25% was considered to indicate excessive irritation and limited biological relevance to the endpoint of sensitisation.

3.2.2.2 ^{3}H-Methyl Thymidine Administration

Five days following the first topical application of the test item or vehicle (Day 6) all mice were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml, specific activity 2.0 Ci/mmol, ARC UK Ltd) giving a total of 20 µCi to each mouse.

3.2.2.3 Observations

Clinical Observations: All animals were observed twice daily on Days 1, 2 and 3 and on a daily basis on Days 4, 5 and 6. Any signs of toxicity or signs of ill health during the test were recorded.

Local Skin Irritation: Local skin irritation was scored daily according to the scale included as Appendix 4.

Bodyweights: The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and Day 6 (prior to termination).

3.2.2.4 Terminal Procedures

Termination: Five hours following the administration of ^{3}HTdR all mice were killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes were excised and processed. For each individual animal 1 ml of PBS was added to the lymph nodes.

Preparation of Single Cell Suspension: A single cell suspension of the lymph node cells for each individual animal was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cells suspension was transferred to a centrifuge tube.

The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The lymph node cells were pelleted at 1400 rpm (approximately 190 *g*) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation: After approximately eighteen hours incubation at approximately 4℃, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 *g*) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe'). ^{3}HTdR incorporation was measured by β-scintillation counting. The "Poly Q^{TM}" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA, USA).

3.3 Statistical Analysis

Data was processed to give group mean values for disintegrations per minute and standard deviations where appropriate. Individual and group mean disintegrations per minute values were assessed for dose response relationships by analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the event of a significant result from the ANOVA, pairwise comparisons were performed between control and treated groups. For homogenous datasets Dunnett's Multiple Comparison test was used and for non-homogenous datasets Dunnett's T3 Multiple Comparison Method was used.

Probability values (p) are presented as follows:

$P<0.001$	***
$P<0.01$	**
$P<0.05$	*
$P\geq0.05$	(not significant)

3.4 Interpretation of Results

The proliferation response of lymph node cells was expressed as the number of radioactive disintegrations per minute per animal and as the ratio of ^{3}HTdR incorporation into lymph node cells of test nodes relative to that recorded for the control nodes (Stimulation Index).

The test item will be regarded as a sensitiser if at least one concentration of the test item results in a threefold or greater increase in ^{3}HTdR incorporation compared to control values. Any test item failing to produce a threefold or greater increase in ^{3}HTdR incorporation will be classified as a "non-sensitiser".

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1 and local skin irritation is given in Table 2. The ear thickness measurements and mean ear thickness changes are given in Table 3.

No signs of systemic toxicity or irritation indicated by an equal to or greater than 25% increase in mean ear thickness were noted. Very slight erythema was noted on both ears on Days 2 to 4.

Based on this information the undiluted test item and the test item at concentrations of 50% and 25% v/v in butanone were selected for the main test.

5.2 Main Test

5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per animal and the stimulation index are given in Table 4.

The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
25	7.47	Positive
50	13.16	Positive
100	13.05	Positive

5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 5 and local skin irritation is given in Table 6. The ear thickness measurements and mean ear thickness changes are given in Table 7.

There were no deaths. No signs of systemic toxicity or irritation indicated by an equal to or greater than 25% increase in mean ear thickness were noted in the test or control animals during the test.

Very slight erythema on the ears was noted on Day 1 in three animals treated with the undiluted test item and persisted in two animals on Day 2. No signs of local skin irritation were noted in the remaining test animals or vehicle control animals during the test.

5.2.3 Bodyweight

Individual bodyweights and bodyweight changes for test and control animals are given in Table 8.

One animal treated with the test item at a concentration of 50% v/v in butanone showed a greater than expected bodyweight loss. Bodyweight changes of the remaining test animals between Day 1 and Day 6 were comparable to those observed in the corresponding control group animals over the same period.

6. CONCLUSION

The test item was considered to be a sensitiser under the conditions of the test.

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 1 Clinical Observations, Bodyweight and Mortality Data – Preliminary Screening Test

Concentration	Animal Number	Bodyweight (g)		Day								
				1		2		3		4	5	6
		Day 1	Day 6	Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
100	S-1	19	20	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 2 Local Skin Irritation – Preliminary Screening Test

Concentration	Animal Number	Local Skin Irritation											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		left	right	left	right	left	right	left	right	left	right	left	right
100	S-1	0	0	1	1	1	1	1	1	0	0	0	0

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 3 Measurement of Ear Thickness and Mean Ear Thickness Changes – Preliminary Screening Test

Concentration	Animal Number	Ear Thickness Measurement (mm)					
		Day 1		Day 3		Day 6	
		pre-dose		post dose			
		left	right	left	right	left	right
100	S-1	0.220	0.215	0.235	0.230	0.240	0.235
overall mean (mm)		0.218		0.233		0.238	
overall mean ear thickness change (%)		na		6.897		9.195	

na = Not applicable

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 4 Individual Disintegrations per Minute and Stimulation Indices

Concentration (% v/v) in butanone	Animal Number	dpm/ Animal[a]	Mean dpm/Animal (Standard Deviation)	Stimulation Index[b]	Result
Vehicle⊕	1-1	1059.75	1610.55 (±522.24)	na	na
	1-2	2102.87			
	1-3	1414.29			
	1-4	1250.99			
	1-5	2224.87			
25	2-1	11161.17	12035.69** (±3017.67)	7.47	Positive
	2-2	14661.98			
	2-3	9085.66			
	2-4	9524.96			
	2-5	15744.69			
50	3-1	27130.23	21194.12*** (±4598.59)	13.16	Positive
	3-2	23022.89			
	3-3	14821.31			
	3-4	19010.66			
	3-5	21985.49			
100	4-1	15835.19	21015.39*** (±6636.64)	13.05	Positive
	4-2	16872.18			
	4-3	19875.62			
	4-4	32417.11			
	4-5	20076.83			

dpm = Disintegrations per minute
a = Total number of lymph nodes per animal is 2
b = Stimulation Index of 3.0 or greater indicates a positive result
⊕ = Control group shared with Project numbers 41205488 and 41205490
na = Not applicable
** = Significantly different from control group $p<0.01$
*** = Significantly different from control group $p<0.001$

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 5 Individual Clinical Observations and Mortality Data

Concentration (% v/v) in butanone	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
Vehicle⊕	1-1	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0
25	2-1	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0
50	3-1	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0
100	4-1	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0

⊕ = Control group shared with Project numbers 41205488 and 41205490
0 = No signs of systemic toxicity

Table 6 Local Skin Irritation – Main Test

Concentration (% v/v) in butanone	Animal Number	Local Skin Irritation											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		left	right	left	right	left	right	left	right	left	right	left	right
Vehicle⊕	1-1	0	0	0	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0	0	0	0
25	2-1	0	0	0	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0	0	0	0
50	3-1	0	0	0	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0	0	0	0
100	4-1	0	0	0	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0	0	0	0
	4-3	1	1	0	0	0	0	0	0	0	0	0	0
	4-4	1	1	1	1	0	0	0	0	0	0	0	0
	4-5	1	1	1	1	0	0	0	0	0	0	0	0

⊕ = Control group shared with Project numbers 41205488 and 41205490

Table 7 Measurement of Ear Thickness and Mean Ear Thickness Changes – Main Test

Concentration (% v/v) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
Vehicle⊕	1-1	0.240	0.240	0.240	0.235	0.230	0.225
	1-2	0.220	0.230	0.230	0.230	0.225	0.230
	1-3	0.230	0.220	0.235	0.245	0.220	0.220
	1-4	0.230	0.230	0.230	0.220	0.230	0.220
	1-5	0.240	0.255	0.230	0.230	0.230	0.235
overall mean (mm)		0.234		0.233		0.227	
overall mean ear thickness change (%)		na		–0.428		–2.998	

Concentration (% v/v) in butanone	[redacted] Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
25	2-1	0.240	0.245	0.250	0.250	0.240	0.245
	2-2	0.250	0.240	0.230	0.230	0.235	0.220
	2-3	0.220	0.240	0.235	0.250	0.230	0.245
	2-4	0.230	0.250	0.240	0.250	0.240	0.255
	2-5	0.220	0.230	0.255	0.235	0.245	0.245
overall mean (mm)		0.237		0.243		0.240	
overall mean ear thickness change (%)		na		2.537		1.480	

⊕ = Control group shared with Project numbers 41205488 and 41205490
na = Not applicable

Table 7 (continued) Measurement of Ear Thickness and Mean Ear Thickness Changes – Main Test

Concentration (% v/v) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
50	3-1	0.245	0.230	0.245	0.240	0.240	0.250
	3-2	0.240	0.250	0.230	0.250	0.240	0.250
	3-3	0.220	0.245	0.250	0.235	0.245	0.250
	3-4	0.230	0.245	0.230	0.245	0.245	0.235
	3-5	0.230	0.240	0.245	0.255	0.245	0.240
overall mean (mm)		0.238		0.243		0.244	
overall mean ear thickness change (%)		na		2.105		2.737	

Concentration	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
100%	4-1	0.250	0.235	0.255	0.225	0.255	0.230
	4-2	0.240	0.245	0.220	0.255	0.230	0.240
	4-3	0.240	0.255	0.225	0.230	0.230	0.235
	4-4	0.220	0.230	0.230	0.225	0.250	0.255
	4-5	0.250	0.240	0.250	0.250	0.250	0.235
overall mean (mm)		0.241		0.237		0.241	
overall mean ear thickness change (%)		na		–1.663		0.208	

na = Not applicable

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 8 Individual Bodyweights and Bodyweight Changes

Concentration (% v/v) in butanone	Animal Number	Bodyweight (g)		Bodyweight Change (g)
		Day 1	Day 6	
Vehicle⊕	1-1	17	18	1
	1-2	20	20	0
	1-3	20	20	0
	1-4	19	18	–1
	1-5	20	22	2
25	2-1	18	19	1
	2-2	18	19	1
	2-3	19	20	1
	2-4	18	20	2
	2-5	18	20	2
50	3-1	21	18	–3
	3-2	19	20	1
	3-3	19	20	1
	3-4	19	20	1
	3-5	20	20	0
100	4-1	22	24	2
	4-2	22	20	–2
	4-3	17	19	–2
	4-4	20	21	1
	4-5	21	19	–2

⊕ = Control group shared with Project numbers 41205488 and 41205490

Appendix 1 Current Positive Control Study for the Local Lymph Node Assay

Introduction. A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The methodology for the LLNA is detailed in the OECD Guideline for the Testing of Chemicals, No. 429, and Method B.42 of Commission Regulation (EC) No. 440/2008. The study described in this document is based on these test methods but has been refined in order to reduce the number of animals required. The reduced LLNA (rLLNA) has been endorsed by the non-Commission members of the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) at its 26th meeting held on 26 – 27 April 2007 at ECVAM, Ispra, Italy.

Test Item:	α-Hexylcinnamaldehyde, tech., 85%
Project number:	41206034
Study dates:	14 November 2012 to 20 November 2012

Methods. A group of five animals was treated with 50 µl (25 µl per ear) of α-Hexylcinnamaldehyde, tech., 85% as a solution in butanone at a concentration of 15% v/v. A further control group of five animals was treated with butanone alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for the treatment group divided by the mean radioactive incorporation of the vehicle control group is as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
15	11.92	Positive

Conclusion. α-Hexylcinnamaldehyde, tech., 85% was considered to be a sensitiser under the conditions of the test.

Appendix 2 Summary of Positive Control Data for the Local Lymph Node Assay

Project Number	Start Date	Finish Date	Test Item	Concentration	Vehicle	Stimulation Index[a]	Classification[b]
41203343	06/06/12	12/06/12	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	3.63	Positive
41203664	22/06/12	28/06/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	ethanol/distilled water 7:3	13.53	Positive
41203665	22/06/12	28/06/12	Phenylacetaldehyde (>90%)	2.5% v/v	propylene glycol	6.48	Positive
41203967	11/07/12	17/07/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone	7.04	Positive
41206031	31/10/12	06/11/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	acetone/olive oil 4:1	6.29	Positive
41206032	31/10/12	06/11/12	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	3.16	Positive
41206033	08/11/12	14/11/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl formamide	4.38	Positive
41206034	14/11/12	20/11/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	butanone	11.92	Positive
41206035	15/12/12	21/12/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	dimethyl sulphoxide	6.80	Positive
41206036	14/12/12	20/12/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	1% pluronic L92 in distilled water	8.31	Positive
41206037	02/01/13	08/01/13	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	ethanol/distilled water 7:3	18.54	Positive
41206038	03/01/13	09/01/13	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone	9.48	Positive
41206039	03/01/13	09/01/13	Phenylacetaldehyde (>90%)	2.5% v/v	propylene glycol	6.48	Positive

a = Ratio of test to control lymphocyte proliferation
b = Stimulation index greater than 3.0 indicates a positive result

Appendix 3 Vehicle Determination Record

Vehicle	Concentration	Method of Preparation	Description of Formulation	Suitability*
acetone/olive oil (4:1)	50% 0.5 ml test item + 0.5 ml vehicle	vortex mixer	na	not suitable for dosing
dimethyl formamide	50% 0.5 ml test item + 0.5 ml vehicle	vortex mixer	na	not suitable for dosing
butanone	50% 0.5 ml test item + 0.5 ml vehicle	vortex mixer	solution	suitable for dosing

* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette

na = Not applicable

Appendix 4 Scale for Erythema

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Appendix 5 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

Harlan Laboratories Ltd
Shardlow Business Park
London Road
Shardlow
Derby
DE72 2GD

TEST TYPE(S)

Analytical/Clinical Chemistry
Environmental Toxicity
Environmental Fate
Mutagenicity
Phys/Chem. Tests
Toxicology

DATE OF INSPECTION
10 July 2012

An inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999).

30/11/12

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

harlan®

[redacted]

LOCAL LYMPH NODE ASSAY IN THE MOUSE

PROJECT NUMBER: 41205488

AUTHOR: A Sanders

STUDY SPONSOR:

[redacted]

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases of this study type at least once every three months.

In addition, general facilities are inspected at least once a year and the results are reported to management.

This report has been audited by the Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

§	24 September 2012	Study Plan Compliance Audit
	17 October 2012	Test Item Preparation
	17 October 2012	Test System Preparation
	17 October 2012	Animal Preparation
	17 October 2012	Dosing
	17 October 2012	Assessment of Response
§	24 January 2013	Draft Report Audit
§	Date of QA Signature	Final Report Audit

..... DATE: .....
For the Quality Assurance Unit*

***Authorised QA Signatures:**
Senior Audit Staff: J G Riley BSc (Hons) MRQA, J M Crowther MIScT MRQA, G Wren ONC MRQA, S Bevan BSc (Hons) MRQA, L Blaney MRQA

GLP COMPLIANCE STATEMENT

With the exception noted below the work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

No analysis was carried out to determine the homogeneity, concentration or stability of the test item formulation. The test item was formulated within two hours of being applied to the test system; it is assumed that the formulation was stable for this duration. This exception is considered not to affect the purpose or integrity of the study.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: .....

A Sanders
Study Director

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[redacted]

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SUMMARY

Introduction. A study was performed to assess the skin sensitisation potential of the test item in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to be compatible with the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 22 July 2010)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

Methods. Following a preliminary screening test in which no clinical signs of toxicity were noted at a concentration of 100%, this concentration was selected as the highest dose investigated in the main test of the Local Lymph Node Assay. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of the undiluted test item or the test item as a solution in butanone at concentrations of 50% or 25% v/v. A further group of five animals was treated with butanone alone. The control group served as a common control with Project numbers 41205486 and 41205490.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
25	9.36	Positive
50	19.02	Positive
100	21.29	Positive

Conclusion. The test item was considered to be a sensitiser under the conditions of the test.

[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION

A study was performed to assess the skin sensitisation potential of the test item in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to be compatible with the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 22 July 2010)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test items that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test item to man.

The study was performed between 07 November 2012 and 05 December 2012.

2. TEST ITEM

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	[redacted]
Description	:	brown liquid
Batch number	:	not supplied
Purity	:	not supplied
Date received	:	23 October 2012
Expiry date	:	not supplied
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test item is the responsibility of the Sponsor.

2.2 Preparation of Test Item

For the purpose of the study, the test item was used undiluted and freshly prepared as a solution in butanone. This vehicle was chosen as it produced the most suitable formulation at the required concentration. The concentrations used are given in the procedure section. The vehicle determination record is included as Appendix 3.

The test item was formulated within two hours of being applied to the test system. It is assumed that the formulation was stable for this duration.

No analysis was conducted to determine the homogeneity, concentration or stability of the test item formulation. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

3. METHODS

3.1 Animals and Animal Husbandry

Female CBA/Ca (CBA/CaOlaHsd) strain mice were supplied by Harlan Laboratories UK Ltd., Oxon, UK. On receipt the animals were randomly allocated to cages. The animals were nulliparous and non-pregnant. After an acclimatisation period of at least five days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were in the weight range of 15 to 23 g, and were eight to twelve weeks old.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (2014C Teklad Global Rodent diet supplied by Harlan Laboratories UK Ltd., Oxon, UK) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25℃ and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled

by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.2 Procedure

3.2.1 Preliminary Screening Test

Using available information regarding the systemic toxicity/irritancy potential of the test item, a preliminary screening test was performed using one mouse. The mouse was treated by daily application of 25 µl of the undiluted test item to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The mouse was observed twice daily on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Local skin irritation was scored daily according to the scale included as Appendix 4. Any clinical signs of toxicity, if present, were also recorded. The bodyweight was recorded on Day 1 (prior to dosing) and on Day 6.

The thickness of each ear was measured using an Oditest micrometer (Dyer, PA), pre-dose on Day 1, post dose on Day 3 and on Day 6. Any changes in the ear thickness were noted. Mean ear thickness changes were calculated between time periods Days 1 to 3 and Days 1 to 6. A mean ear thickness increase of equal to or greater than 25% was considered to indicate excessive irritation and limited biological relevance to the endpoint of sensitisation.

3.2.2 Main Test

3.2.2.1 Test Item Administration

Groups of five mice were treated with the undiluted test item or the test item at concentrations of 50% or 25% v/v in butanone. The preliminary screening test suggested that the test item would not produce systemic toxicity or excessive local irritation at the highest suitable concentration. The mice were treated by daily application of 25 µl of the appropriate concentration of the test item to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test item formulation was administered using an automatic micropipette and spread over the dorsal surface of the ear using the tip of the pipette.

A further group of five mice received the vehicle alone in the same manner. The control group served as a common control with Project numbers 41205486 and 41205490.

The thickness of each ear of each animal was measured using an Oditest micrometer (Dyer, PA), on Days 1, 3 and 6. Any changes in the ear thickness were noted. Mean ear thickness changes were calculated between Days 1 to 3 and Days 1 to 6. A mean ear thickness increase of equal to or greater than 25% was considered to indicate excessive irritation and limited biological relevance to the endpoint of sensitisation.

3.2.2.2 ^{3}H-Methyl Thymidine Administration

Five days following the first topical application of the test item or vehicle (Day 6) all mice were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml, specific activity 2.0 Ci/mmol, ARC UK Ltd) giving a total of 20 µCi to each mouse.

3.2.2.3 Observations

Clinical Observations: All animals were observed twice daily on Days 1, 2 and 3 and on a daily basis on Days 4, 5 and 6. Any signs of toxicity or signs of ill health during the test were recorded.

Local Skin Irritation: Local skin irritation was scored daily according to the scale included as Appendix 4.

Bodyweights: The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and Day 6 (prior to termination).

3.2.2.4 Terminal Procedures

Termination: Five hours following the administration of ^{3}HTdR all mice were killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes were excised and processed. For each individual animal 1 ml of PBS was added to the lymph nodes.

Preparation of Single Cell Suspension: A single cell suspension of the lymph node cells for each individual animal was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cells suspension was transferred to a centrifuge tube.

The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The lymph node cells were pelleted at 1400 rpm (approximately 190 *g*) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation: After approximately eighteen hours incubation at approximately 4℃, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 *g*) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe'). ^{3}HTdR incorporation was measured by β-scintillation counting. The "Poly Q™" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA, USA).

3.3 Statistical Analysis

Data was processed to give group mean values for disintegrations per minute and standard deviations where appropriate. Individual and group mean disintegrations per minute values were assessed for dose response relationships by analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the event of a significant result from the ANOVA, pairwise comparisons were performed between control and treated groups. For homogenous datasets Dunnett's Multiple Comparison test was used and for non-homogenous datasets Dunnett's T3 Multiple Comparison Method was used.

Probability values (p) are presented as follows:

$P<0.001$	***
$P<0.01$	**
$P<0.05$	*
$P\geq0.05$	(not significant)

3.4 Interpretation of Results

The proliferation response of lymph node cells was expressed as the number of radioactive disintegrations per minute per animal and as the ratio of ^{3}HTdR incorporation into lymph node cells of test nodes relative to that recorded for the control nodes (Stimulation Index).

The test item will be regarded as a sensitiser if at least one concentration of the test item results in a threefold or greater increase in ^{3}HTdR incorporation compared to control values. Any test item failing to produce a threefold or greater increase in ^{3}HTdR incorporation will be classified as a "non-sensitiser".

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1 and local skin irritation is given in Table 2. The ear thickness measurements and mean ear thickness changes are given in Table 3.

No signs of systemic toxicity or irritation indicated by an equal to or greater than 25% increase in mean ear thickness were noted. Very slight erythema was noted on both ears on Days 3 and 4.

Based on this information the undiluted test item and the test item at concentrations of 50% and 25% v/v in butanone were selected for the main test.

5.2 Main Test

5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per animal and the stimulation index are given in Table 4.

The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
25	9.36	Positive
50	19.02	Positive
100	21.29	Positive

5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 5 and local skin irritation is given in Table 6. The ear thickness measurements and mean ear thickness changes are given in Table 7.

No signs of systemic toxicity, visual local skin irritation or irritation indicated by an equal to or greater than 25% increase in mean ear thickness were noted.

5.2.3 Bodyweight

Individual bodyweights and bodyweight changes for test and control animals are given in Table 8.

Bodyweight changes of the test animals between Day 1 and Day 6 were comparable to those observed in the corresponding control group animals over the same period.

6. CONCLUSION

The test item was considered to be a sensitiser under the conditions of the test.

[redacted]: LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 1 Clinical Observations, Bodyweight and Mortality Data – Preliminary Screening Test

Concentration	Animal Number	Bodyweight (g)		Day								
				1		2		3		4	5	6
		Day 1	Day 6	Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
100	S-1	19	20	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

[redacted]: LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 2 Local Skin Irritation – Preliminary Screening Test

Concentration	Animal Number	Local Skin Irritation											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		left	right	left	right	left	right	left	right	left	right	left	right
100	S-1	0	0	0	0	1	1	1	1	0	0	0	0

[redacted]: LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 3 Measurement of Ear Thickness and Mean Ear Thickness Changes – Preliminary Screening Test

Concentration	Animal Number	Ear Thickness Measurement (mm)					
		Day 1		Day 3		Day 6	
		pre-dose		post dose			
		left	right	left	right	left	right
100	S-1	0.225	0.230	0.240	0.240	0.245	0.240
overall mean (mm)		0.228		0.240		0.243	
overall mean ear thickness change (%)		na		5.495		6.593	

na = Not applicable

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 4 Individual Disintegrations per Minute and Stimulation Indices

Concentration (% v/v) in butanone	Animal Number	dpm/ Animal[a]	Mean dpm/Animal (Standard Deviation)	Stimulation Index [b]	Result
Vehicle⊕	1-1	1059.75	1610.55 (±522.24)	na	na
	1-2	2102.87			
	1-3	1414.29			
	1-4	1250.99			
	1-5	2224.87			
25	2-1	10472.37	15081.41** (±2793.65)	9.36	Positive
	2-2	15645.08			
	2-3	16105.04			
	2-4	18014.20			
	2-5	15170.38			
50	3-1	28766.81	30629.99*** (±2826.90)	19.02	Positive
	3-2	26579.94			
	3-3	32844.88			
	3-4	32895.44			
	3-5	32062.88			
100	4-1	37179.77	34291.05** (±6926.05)	21.29	Positive
	4-2	36662.81			
	4-3	43114.41			
	4-4	26567.93			
	4-5	27930.34			

dpm = Disintegrations per minute
a = Total number of lymph nodes per animal is 2
b = Stimulation Index of 3.0 or greater indicates a positive result
⊕ = Control group shared with Project numbers 41205486 and 41205490
na = Not applicable
** = Significantly different from control group $p<0.01$
*** = Significantly different from control group $p<0.001$

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 5 Individual Clinical Observations and Mortality Data

Concentration (% v/v) in butanone	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
Vehicle⊕	1-1	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0
25	2-1	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0
50	3-1	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0
100	4-1	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0

⊕ = Control group shared with Project numbers 41205486 and 41205490
0 = No signs of systemic toxicity

Table 6 Local Skin Irritation – Main Test

Concentration (% v/v) in butanone	Animal Number	Local Skin Irritation											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		left	right	left	right	left	right	left	right	left	right	left	right
Vehicle⊕	1-1	0	0	0	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0	0	0	0
25	2-1	0	0	0	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0	0	0	0
50	3-1	0	0	0	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0	0	0	0
100	4-1	0	0	0	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0	0	0	0

⊕ = Control group shared with Project numbers 41205486 and 41205490

Table 7 Measurement of Ear Thickness and Mean Ear Thickness Changes – Main Test

Concentration (% v/v) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
Vehicle⊕	1-1	0.240	0.240	0.240	0.235	0.230	0.225
	1-2	0.220	0.230	0.230	0.230	0.225	0.230
	1-3	0.230	0.220	0.235	0.245	0.220	0.220
	1-4	0.230	0.230	0.230	0.220	0.230	0.220
	1-5	0.240	0.255	0.230	0.230	0.230	0.235
overall mean (mm)		0.234		0.233		0.227	
overall mean ear thickness change (%)		na		–0.428		–2.998	

Concentration (% v/v) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
25	2-1	0.220	0.215	0.240	0.220	0.235	0.230
	2-2	0.235	0.220	0.230	0.240	0.240	0.240
	2-3	0.240	0.230	0.250	0.220	0.245	0.235
	2-4	0.225	0.230	0.235	0.220	0.240	0.245
	2-5	0.220	0.215	0.240	0.240	0.235	0.240
overall mean (mm)		0.225		0.234		0.239	
overall mean ear thickness change (%)		na		3.778		6.000	

⊕ = Control group shared with Project numbers 41205486 and 41205490
na = Not applicable

Table 7 (continued) Measurement of Ear Thickness and Mean Ear Thickness Changes – Main Test

Concentration (% v/v) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
50	3-1	0.235	0.220	0.220	0.250	0.235	0.255
	3-2	0.225	0.230	0.250	0.235	0.250	0.245
	3-3	0.230	0.220	0.220	0.220	0.235	0.245
	3-4	0.230	0.225	0.240	0.220	0.235	0.240
	3-5	0.235	0.240	0.240	0.240	0.225	0.240
overall mean (mm)		0.229		0.234		0.241	
overall mean ear thickness change (%)		na		1.965		5.022	

Concentration	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
100%	4-1	0.215	0.220	0.205	0.210	0.215	0.225
	4-2	0.210	0.215	0.245	0.225	0.230	0.235
	4-3	0.210	0.210	0.205	0.205	0.220	0.230
	4-4	0.225	0.220	0.225	0.230	0.230	0.230
	4-5	0.220	0.225	0.240	0.240	0.235	0.240
overall mean (mm)		0.217		0.223		0.229	
overall mean ear thickness change (%)		na		2.765		5.530	

na = Not applicable

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 8 Individual Bodyweights and Bodyweight Changes

Concentration (% v/v) in butanone	Animal Number	Bodyweight (g)		Bodyweight Change (g)
		Day 1	Day 6	
Vehicle⊕	1-1	17	18	1
	1-2	20	20	0
	1-3	20	20	0
	1-4	19	18	–1
	1-5	20	22	2
25	2-1	19	20	1
	2-2	20	21	1
	2-3	19	20	1
	2-4	20	22	2
	2-5	21	20	–1
50	3-1	21	20	–1
	3-2	19	22	3
	3-3	21	22	1
	3-4	21	21	0
	3-5	19	20	1
100	4-1	19	19	0
	4-2	19	21	2
	4-3	20	20	0
	4-4	18	18	0
	4-5	21	21	0

⊕ = Control group shared with Project numbers 41205486 and 41205490

Appendix 1 Current Positive Control Study for the Local Lymph Node Assay

Introduction. A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The methodology for the LLNA is detailed in the OECD Guideline for the Testing of Chemicals, No. 429, and Method B.42 of Commission Regulation (EC) No. 440/2008. The study described in this document is based on these test methods but has been refined in order to reduce the number of animals required. The reduced LLNA (rLLNA) has been endorsed by the non-Commission members of the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) at its 26th meeting held on 26 – 27 April 2007 at ECVAM, Ispra, Italy.

Test Item:	α-Hexylcinnamaldehyde, tech., 85%
Project number:	41206034
Study dates:	14 November 2012 to 20 November 2012

Methods. A group of five animals was treated with 50 µl (25 µl per ear) of α-Hexylcinnamaldehyde, tech., 85% as a solution in butanone at a concentration of 15% v/v. A further control group of five animals was treated with butanone alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for the treatment group divided by the mean radioactive incorporation of the vehicle control group is as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
15	11.92	Positive

Conclusion. α-Hexylcinnamaldehyde, tech., 85% was considered to be a sensitiser under the conditions of the test.

Appendix 2 Summary of Positive Control Data for the Local Lymph Node Assay

Project Number	Start Date	Finish Date	Test Item	Concentration	Vehicle	Stimulation Index[a]	Classification[b]
41201832	06/04/12	12/04/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	acetone/olive oil 4:1	5.76	Positive
41202697	16/05/12	22/05/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl formamide	5.74	Positive
41202698	16/05/12	22/05/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	butanone	4.57	Positive
41202699	16/05/12	22/05/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	dimethyl sulphoxide	5.45	Positive
41203342	06/06/12	12/06/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	1% pluronic L92 in distilled water	7.20	Positive
41203343	06/06/12	12/06/12	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	3.63	Positive
41203664	22/06/12	28/06/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	ethanol/distilled water 7:3	13.53	Positive
41203665	22/06/12	28/06/12	Phenylacetaldehyde (>90%)	2.5% v/v	propylene glycol	6.48	Positive
41203967	11/07/12	17/07/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone	7.04	Positive
41206031	31/10/12	06/11/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	acetone/olive oil 4:1	6.29	Positive
41206032	31/10/12	06/11/12	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	3.16	Positive
41206033	08/11/12	14/11/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl formamide	4.38	Positive
41206034	14/11/12	20/11/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	butanone	11.92	Positive

a = Ratio of test to control lymphocyte proliferation
b = Stimulation index greater than 3.0 indicates a positive result

Appendix 3 Vehicle Determination Record

Vehicle	Concentration	Method of Preparation	Description of Formulation	Suitability*
acetone/olive oil (4:1)	50% 0.5 ml test item + 0.5 ml vehicle	vortex mixer	na	not suitable for dosing
dimethyl formamide	50% 0.5 ml test item + 0.5 ml vehicle	vortex mixer	na	not suitable for dosing
butanone	50% 0.5 ml test item + 0.5 ml vehicle	vortex mixer	solution	suitable for dosing

* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette

na = Not applicable

[redacted]: LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 4 Scale for Erythema

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Appendix 5 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

Harlan Laboratories Ltd
Shardlow Business Park
London Road
Shardlow
Derby
DE72 2GD

TEST TYPE(S)

Analytical/Clinical Chemistry
Environmental Toxicity
Environmental Fate
Mutagenicity
Phys/Chem. Tests
Toxicology

DATE OF INSPECTION
10 July 2012

An inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999).

30/11/12

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA

harlan®

[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

PROJECT NUMBER: 41205490

AUTHOR: A Sanders

STUDY SPONSOR:

[redacted]

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QUALITY ASSURANCE REPORT

This study type is classed as short-term. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases of this study type at least once every three months.

In addition, general facilities are inspected at least once a year and the results are reported to management.

This report has been audited by the Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

§	24 September 2012	Study Plan Compliance Audit
	17 October 2012	Test Item Preparation
	17 October 2012	Test System Preparation
	17 October 2012	Animal Preparation
	17 October 2012	Dosing
	17 October 2012	Assessment of Response
§	24 January 2013	Draft Report Audit
§	Date of QA Signature	Final Report Audit

..... DATE: .....
For the Quality Assurance Unit*

***Authorised QA Signatures:**
Senior Audit Staff: J G Riley BSc (Hons) MRQA, J M Crowther MIScT MRQA, G Wren ONC MRQA, S Bevan BSc (Hons) MRQA, L Blaney MRQA

GLP COMPLIANCE STATEMENT

With the exception noted below the work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

No analysis was carried out to determine the homogeneity, concentration or stability of the test item formulation. The test item was formulated within two hours of being applied to the test system; it is assumed that the formulation was stable for this duration. This exception is considered not to affect the purpose or integrity of the study.

This report fully and accurately reflects the procedures used and data generated.

..... DATE: .....

A Sanders
Study Director

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[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

SUMMARY

Introduction. A study was performed to assess the skin sensitisation potential of the test item in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to be compatible with the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 22 July 2010)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

Methods. Following a preliminary screening test in which no clinical signs of toxicity were noted at a concentration of 50% w/w, this concentration was selected as the highest dose investigated in the main test of the Local Lymph Node Assay. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of the test item as a solution in butanone at concentrations of 50%, 25% or 10% w/w. A further group of five animals was treated with butanone alone. The control group served as a common control with Project numbers 41205486 and 41205488.

Results. The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% w/w) in butanone	Stimulation Index	Result
10	3.00	Negative
25	5.10	Positive
50	8.65	Positive

The concentration of test item expected to cause a 3 fold increase in ^{3}HTdR incorporation (EC_3 value) was calculated to be 10%.

Conclusion. The test item was considered to be a sensitiser under the conditions of the test.

[redacted]:

LOCAL LYMPH NODE ASSAY IN THE MOUSE

1. INTRODUCTION

A study was performed to assess the skin sensitisation potential of the test item in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to be compatible with the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 22 July 2010)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008

The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test items that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test item to man.

The study was performed between 07 November 2012 and 05 December 2012.

2. TEST ITEM

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	[redacted]
Description	:	beige waxy solid
Batch number	:	[redacted]
Purity	:	not supplied
Date received	:	23 October 2012
Expiry date	:	23 October 2013
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test item is the responsibility of the Sponsor.

2.2 Preparation of Test Item

For the purpose of the study, the test item was freshly prepared as a solution in butanone. This vehicle was chosen as it produced the highest concentration that was suitable for dosing. The concentrations used are given in the procedure section. The vehicle determination record is included as Appendix 3.

The test item was formulated within two hours of being applied to the test system. It is assumed that the formulation was stable for this duration.

No analysis was conducted to determine the homogeneity, concentration or stability of the test item formulation. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

3. METHODS

3.1 Animals and Animal Husbandry

Female CBA/Ca (CBA/CaOlaHsd) strain mice were supplied by Harlan Laboratories UK Ltd., Oxon, UK. On receipt the animals were randomly allocated to cages. The animals were nulliparous and non-pregnant. After an acclimatisation period of at least five days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were in the weight range of 15 to 23 g, and were eight to twelve weeks old.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (2014C Teklad Global Rodent diet supplied by Harlan Laboratories UK Ltd., Oxon, UK) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25°C and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled

by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

3.2 Procedure

3.2.1 Preliminary Screening Test

Using available information regarding the systemic toxicity/irritancy potential of the test item, a preliminary screening test was performed using one mouse. The mouse was treated by daily application of 25 µl of the test item at a concentration of 50% w/w in butanone, to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The mouse was observed twice daily on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Local skin irritation was scored daily according to the scale included as Appendix 4. Any clinical signs of toxicity, if present, were also recorded. The bodyweight was recorded on Day 1 (prior to dosing) and on Day 6.

The thickness of each ear was measured using an Oditest micrometer (Dyer, PA), pre-dose on Day 1, post dose on Day 3 and on Day 6. Any changes in the ear thickness were noted. Mean ear thickness changes were calculated between time periods Days 1 to 3 and Days 1 to 6. A mean ear thickness increase of equal to or greater than 25% was considered to indicate excessive irritation and limited biological relevance to the endpoint of sensitisation.

3.2.2 Main Test

3.2.2.1 Test Item Administration

Groups of five mice were treated with the test item at concentrations of 50%, 25% or 10% w/w in butanone. The preliminary screening test suggested that the test item would not produce systemic toxicity or excessive local irritation at the highest suitable concentration. The mice were treated by daily application of 25 µl of the appropriate concentration of the test item to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The test item formulation was administered using an automatic micropipette and spread over the dorsal surface of the ear using the tip of the pipette.

A further group of five mice received the vehicle alone in the same manner. The control group served as a common control with Project numbers 41205486 and 41205488.

The thickness of each ear of each animal was measured using an Oditest micrometer (Dyer, PA), on Days 1, 3 and 6. Any changes in the ear thickness were noted. Mean ear thickness changes were calculated between Days 1 to 3 and Days 1 to 6. A mean ear thickness increase of equal to or greater than 25% was considered to indicate excessive irritation and limited biological relevance to the endpoint of sensitisation.

3.2.2.2 ^{3}H-Methyl Thymidine Administration

Five days following the first topical application of the test item or vehicle (Day 6) all mice were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing ^{3}H-methyl thymidine (^{3}HTdR:80µCi/ml, specific activity 2.0 Ci/mmol, ARC UK Ltd) giving a total of 20 µCi to each mouse.

3.2.2.3 Observations

Clinical Observations: All animals were observed twice daily on Days 1, 2 and 3 and on a daily basis on Days 4, 5 and 6. Any signs of toxicity or signs of ill health during the test were recorded.

Local Skin Irritation: Local skin irritation was scored daily according to the scale included as Appendix 4.

Bodyweights: The bodyweight of each mouse was recorded on Day 1 (prior to dosing) and Day 6 (prior to termination).

3.2.2.4 Terminal Procedures

Termination: Five hours following the administration of ^{3}HTdR all mice were killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes were excised and processed. For each individual animal 1 ml of PBS was added to the lymph nodes.

Preparation of Single Cell Suspension: A single cell suspension of the lymph node cells for each individual animal was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cells suspension was transferred to a centrifuge tube.

The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The lymph node cells were pelleted at 1400 rpm (approximately 190 *g*) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

Determination of ^{3}HTdR Incorporation: After approximately eighteen hours incubation at approximately 4℃, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 *g*) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe'). ^{3}HTdR incorporation was measured by β-scintillation counting. The "Poly Q^{TM}" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA, USA).

3.3 Statistical Analysis

Data was processed to give group mean values for disintegrations per minute and standard deviations where appropriate. Individual and group mean disintegrations per minute values were assessed for dose response relationships by analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the event of a significant result from the ANOVA, pairwise comparisons were performed between control and treated groups. For homogenous datasets Dunnett's Multiple Comparison test was used and for non-homogenous datasets Dunnett's T3 Multiple Comparison Method was used.

Probability values (p) are presented as follows:

$P<0.001$	***
$P<0.01$	**
$P<0.05$	*
$P\geq0.05$	(not significant)

3.4 Interpretation of Results

The proliferation response of lymph node cells was expressed as the number of radioactive disintegrations per minute per animal and as the ratio of ^{3}HTdR incorporation into lymph node cells of test nodes relative to that recorded for the control nodes (Stimulation Index).

The test item will be regarded as a sensitiser if at least one concentration of the test item results in a threefold or greater increase in ^{3}HTdR incorporation compared to control values. Any test item failing to produce a threefold or greater increase in ^{3}HTdR incorporation will be classified as a "non-sensitiser".

The EC_3 value was also calculated. The EC_3 value is the concentration of test item expected to cause a 3 fold increase in ^{3}HTdR incorporation. The equation used for the calculation of EC_3 is:

$EC_3 = c + [[(3–d)/(b–d)] \times (a–c)]$

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Harlan Laboratories Ltd, Shardlow, UK archives for five years, after which instructions will be sought as to further retention or disposal.

a = lowest concentration giving stimulation index >3
b = actual stimulation index caused by 'a'
c = highest concentration failing to produce a stimulation index of 3
d = actual stimulation index caused by 'c'

5. RESULTS

5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1 and local skin irritation is given in Table 2. The ear thickness measurements and mean ear thickness changes are given in Table 3.

No signs of systemic toxicity, visual local skin irritation or irritation indicated by an equal to or greater than 25% increase in mean ear thickness were noted.

Based on this information the dose levels selected for the main test were 50%, 25% and 10% w/w in butanone.

5.2 Main Test

5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per animal and the stimulation index are given in Table 4.

The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

Concentration (% w/w) in butanone	Stimulation Index	Result
10	3.00	Negative
25	5.10	Positive
50	8.65	Positive

5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 5 and local skin irritation is given in Table 6. The ear thickness measurements and mean ear thickness changes are given in Table 7.

There were no deaths. No signs of systemic toxicity, visual local skin irritation or irritation indicated by an equal to or greater than 25% increase in mean ear thickness were noted.

5.2.3 Bodyweight

Individual bodyweights and bodyweight changes for test and control animals are given in Table 8.

Bodyweight changes of the test animals between Day 1 and Day 6 were comparable to those observed in the corresponding control group animals over the same period.

6. CALCULATION OF EC_3 VALUE

$EC_3 = c + [[(3–d)/(b–d)] \times (a–c)]$

a = 25
b = 5.10
c = 10
d = 3.00

$EC_3 = + [[(3–3.00)/(5.10–3.00)] \times (25–10)] = 10$

The concentration of test item expected to cause a 3 fold increase in ^{3}HTdR incorporation (EC_3 value) was calculated to be 10%.

7. CONCLUSION

The test item was considered to be a sensitiser under the conditions of the test.

a = lowest concentration giving stimulation index >3
b = actual stimulation index caused by 'a'
c = highest concentration failing to produce a stimulation index of 3
d = actual stimulation index caused by 'c'

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 1 Clinical Observations, Bodyweight and Mortality Data – Preliminary Screening Test

Concentration (% w/w) in butanone	Animal Number	Bodyweight (g)		Day								
				1		2		3		4	5	6
		Day 1	Day 6	Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
50	S-1	21	20	0	0	0	0	0	0	0	0	0

0 = No signs of systemic toxicity

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 2 Local Skin Irritation – Preliminary Screening Test

Concentration (% w/w) in butanone	Animal Number	Local Skin Irritation											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		left	right	left	right	left	right	left	right	left	right	left	right
50	S-1	0	0	0	0	0	0	0	0	0	0	0	0

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 3 Measurement of Ear Thickness and Mean Ear Thickness Changes – Preliminary Screening Test

Concentration (% w/w) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1		Day 3		Day 6	
		pre-dose		post dose			
		left	right	left	right	left	right
50	S-1	0.235	0.225	0.240	0.235	0.250	0.235
overall mean (mm)		0.230		0.238		0.243	
overall mean ear thickness change (%)		na		3.261		5.435	

na = Not applicable

[redacted]: LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 4 Individual Disintegrations per Minute and Stimulation Indices

Concentration (% w/w) in butanone	Animal Number	dpm/ Animal[a]	Mean dpm/Animal (Standard Deviation)	Stimulation Index[b]	Result
Vehicle⊕	1-1	1059.75	1610.55 (±522.24)	na	na
	1-2	2102.87			
	1-3	1414.29			
	1-4	1250.99			
	1-5	2224.87			
10	2-1	4787.21	4824.79 (±2063.08)	3.00	Negative
	2-2	7307.01			
	2-3	3602.56			
	2-4	2144.44			
	2-5	6302.71			
25	3-1	9088.77	8206.20** (±1959.71)	5.10	Positive
	3-2	10468.22			
	3-3	5820.91			
	3-4	6505.88			
	3-5	9147.24			
50	4-1	20170.82	13930.10*** (±4156.72)	8.65	Positive
	4-2	13839.78			
	4-3	9372.59			
	4-4	15148.47			
	4-5	11118.86			

dpm = Disintegrations per minute
a = Total number of lymph nodes per animal is 2
b = Stimulation Index of 3.0 or greater indicates a positive result
⊕ = Control group shared with Project numbers 41205486 and 41205488
na = Not applicable
** = Significantly different from control group $p<0.01$
*** = Significantly different from control group $p<0.001$

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 5 Individual Clinical Observations and Mortality Data

Concentration (% w/w) in butanone	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre-Dose	Post Dose	Pre-Dose	Post Dose	Pre-Dose	Post Dose			
Vehicle⊕	1-1	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0
10	2-1	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0
25	3-1	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0
50	4-1	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0

⊕ = Control group shared with Project numbers 41205486 and 41205488
0 = No signs of systemic toxicity

Table 6 Local Skin Irritation – Main Test

Concentration (% w/w) in butanone	Animal Number	Local Skin Irritation											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		left	right	left	right	left	right	left	right	left	right	left	right
Vehicle⊕	1-1	0	0	0	0	0	0	0	0	0	0	0	0
	1-2	0	0	0	0	0	0	0	0	0	0	0	0
	1-3	0	0	0	0	0	0	0	0	0	0	0	0
	1-4	0	0	0	0	0	0	0	0	0	0	0	0
	1-5	0	0	0	0	0	0	0	0	0	0	0	0
10	2-1	0	0	0	0	0	0	0	0	0	0	0	0
	2-2	0	0	0	0	0	0	0	0	0	0	0	0
	2-3	0	0	0	0	0	0	0	0	0	0	0	0
	2-4	0	0	0	0	0	0	0	0	0	0	0	0
	2-5	0	0	0	0	0	0	0	0	0	0	0	0
25	3-1	0	0	0	0	0	0	0	0	0	0	0	0
	3-2	0	0	0	0	0	0	0	0	0	0	0	0
	3-3	0	0	0	0	0	0	0	0	0	0	0	0
	3-4	0	0	0	0	0	0	0	0	0	0	0	0
	3-5	0	0	0	0	0	0	0	0	0	0	0	0
50	4-1	0	0	0	0	0	0	0	0	0	0	0	0
	4-2	0	0	0	0	0	0	0	0	0	0	0	0
	4-3	0	0	0	0	0	0	0	0	0	0	0	0
	4-4	0	0	0	0	0	0	0	0	0	0	0	0
	4-5	0	0	0	0	0	0	0	0	0	0	0	0

⊕ = Control group shared with Project numbers 41205486 and 41205488

Table 7 Measurement of Ear Thickness and Mean Ear Thickness Changes – Main Test

Concentration (% w/w) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
Vehicle⊕	1-1	0.240	0.240	0.240	0.235	0.230	0.225
	1-2	0.220	0.230	0.230	0.230	0.225	0.230
	1-3	0.230	0.220	0.235	0.245	0.220	0.220
	1-4	0.230	0.230	0.230	0.220	0.230	0.220
	1-5	0.240	0.255	0.230	0.230	0.230	0.235
overall mean (mm)		0.234		0.233		0.227	
overall mean ear thickness change (%)		na		–0.428		–2.998	

Concentration (% w/w) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
10	2-1	0.210	0.220	0.235	0.220	0.225	0.205
	2-2	0.210	0.220	0.240	0.240	0.225	0.230
	2-3	0.220	0.215	0.235	0.220	0.230	0.230
	2-4	0.225	0.220	0.220	0.220	0.235	0.225
	2-5	0.215	0.210	0.235	0.225	0.220	0.210
overall mean (mm)		0.217		0.229		0.224	
overall mean ear thickness change (%)		na		5.774		3.233	

⊕ = Control group shared with Project numbers 41205486 and 41205488
na = Not applicable

Table 7 (continued) Measurement of Ear Thickness and Mean Ear Thickness Changes – Main Test

Concentration (% w/w) in butanone	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
25	3-1	0.220	0.215	0.235	0.240	0.235	0.240
	3-2	0.230	0.220	0.230	0.230	0.245	0.235
	3-3	0.215	0.215	0.225	0.220	0.245	0.230
	3-4	0.230	0.235	0.240	0.245	0.230	0.235
	3-5	0.215	0.210	0.210	0.215	0.230	0.210
overall mean (mm)		0.221		0.229		0.234	
overall mean ear thickness change (%)		na		3.855		5.896	

Concentration	Animal Number	Ear Thickness Measurement (mm)					
		Day 1 pre-dose		Day 3 post dose		Day 6	
		left	right	left	right	left	right
50%	4-1	0.215	0.220	0.240	0.240	0.245	0.245
	4-2	0.215	0.215	0.230	0.240	0.240	0.230
	4-3	0.225	0.220	0.240	0.215	0.250	0.225
	4-4	0.225	0.215	0.205	0.220	0.220	0.230
	4-5	0.230	0.230	0.240	0.250	0.250	0.250
overall mean (mm)		0.221		0.232		0.239	
overall mean ear thickness change (%)		na		4.977		7.919	

na = Not applicable

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 8 Individual Bodyweights and Bodyweight Changes

Concentration (% w/w) in butanone	Animal Number	Bodyweight (g)		Bodyweight Change (g)
		Day 1	Day 6	
Vehicle⊕	1-1	17	18	1
	1-2	20	20	0
	1-3	20	20	0
	1-4	19	18	–1
	1-5	20	22	2
10	2-1	19	18	–1
	2-2	18	19	1
	2-3	19	20	1
	2-4	17	17	0
	2-5	18	20	2
25	3-1	20	21	1
	3-2	17	18	1
	3-3	21	21	0
	3-4	20	19	–1
	3-5	20	21	1
50	4-1	20	21	1
	4-2	18	18	0
	4-3	21	20	–1
	4-4	19	20	1
	4-5	19	20	1

⊕ = Control group shared with Project numbers 41205486 and 41205488

Appendix 1 Current Positive Control Study for the Local Lymph Node Assay

Introduction. A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The methodology for the LLNA is detailed in the OECD Guideline for the Testing of Chemicals, No. 429, and Method B.42 of Commission Regulation (EC) No. 440/2008. The study described in this document is based on these test methods but has been refined in order to reduce the number of animals required. The reduced LLNA (rLLNA) has been endorsed by the non-Commission members of the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) at its 26th meeting held on 26 – 27 April 2007 at ECVAM, Ispra, Italy.

Test Item:	α-Hexylcinnamaldehyde, tech., 85%
Project number:	41206034
Study dates:	14 November 2012 to 20 November 2012

Methods. A group of five animals was treated with 50 µl (25 µl per ear) of α-Hexylcinnamaldehyde, tech., 85% as a solution in butanone at a concentration of 15% v/v. A further control group of five animals was treated with butanone alone.

Results. The Stimulation Index expressed as the mean radioactive incorporation for the treatment group divided by the mean radioactive incorporation of the vehicle control group is as follows:

Concentration (% v/v) in butanone	Stimulation Index	Result
15	11.92	Positive

Conclusion. α-Hexylcinnamaldehyde, tech., 85% was considered to be a sensitiser under the conditions of the test.

Appendix 2 Summary of Positive Control Data for the Local Lymph Node Assay

Project Number	Start Date	Finish Date	Test Item	Concentration	Vehicle	Stimulation Index[a]	Classification[b]
41203343	06/06/12	12/06/12	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	3.63	Positive
41203664	22/06/12	28/06/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	ethanol/distilled water 7:3	13.53	Positive
41203665	22/06/12	28/06/12	Phenylacetaldehyde (>90%)	2.5% v/v	propylene glycol	6.48	Positive
41203967	11/07/12	17/07/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone	7.04	Positive
41206031	31/10/12	06/11/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	acetone/olive oil 4:1	6.29	Positive
41206032	31/10/12	06/11/12	α-Hexylcinnamaldehyde, tech., 85%	50% v/v	cottonseed oil	3.16	Positive
41206033	08/11/12	14/11/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	dimethyl formamide	4.38	Positive
41206034	14/11/12	20/11/12	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	butanone	11.92	Positive
41206035	15/12/12	21/12/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	dimethyl sulphoxide	6.80	Positive
41206036	14/12/12	20/12/12	α-Hexylcinnamaldehyde, tech., 85%	25% v/v	1% pluronic L92 in distilled water	8.31	Positive
41206037	02/01/13	08/01/13	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	ethanol/distilled water 7:3	18.54	Positive
41206038	03/01/13	09/01/13	α-Hexylcinnamaldehyde, tech., 85%	15% v/v	acetone	9.48	Positive
41206039	03/01/13	09/01/13	Phenylacetaldehyde (>90%)	2.5% v/v	propylene glycol	6.48	Positive

a = Ratio of test to control lymphocyte proliferation
b = Stimulation index greater than 3.0 indicates a positive result

[redacted] : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Appendix 3 Vehicle Determination Record

Vehicle	Concentration	Method of Preparation	Description of Formulation	Suitability*
acetone/olive oil (4:1)	50% 0.5 g test item + 0.5 g vehicle	1, 2	na	not suitable for dosing
dimethyl formamide	50% 0.5 g test item + 0.5 g vehicle	1, 2	na	not suitable for dosing
butanone	50% 0.5 g test item + 0.5 g vehicle	1, 2	solution	suitable for dosing

* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette
na = Not applicable
1 = Vortex mixer
2 = Heated in water bath at 40°C for 8 minutes

Appendix 4 Scale for Erythema

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4

Appendix 5 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

TEST FACILITY

Harlan Laboratories Ltd
Shardlow Business Park
London Road
Shardlow
Derby
DE72 2GD

TEST TYPE(S)

Analytical/Clinical
Chemistry
Environmental Toxicity
Environmental Fate
Mutagenicity
Phys/Chem. Tests
Toxicology

DATE OF INSPECTION
10 July 2012

An inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999)

30/11/12

Dr. Andrew J. Gray
Head, UK GLP Monitoring Authority

MHRA